

Abundance of Drug Transporters in the Human Kidney Cortex as Quantified by Quantitative Targeted Proteomics[§]

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ABSTRACT

Protein expression of renal uptake and efflux transporters was quantified by quantitative targeted proteomics using the surrogate peptide approach. Renal uptake transporters assessed in this study included organic anion transporters (OAT1–OAT4), organic cation transporter 2 (OCT2), organic/carnitine cation transporters (OCTN1 and OCTN2), and sodium-glucose transporter 2 (SGLT2); efflux transporters included P-glycoprotein, breast cancer resistance protein, multidrug resistance proteins (MRP2 and MRP4), and multidrug and toxin extrusion proteins (MATE1 and MATE2-K). Total membrane was isolated from the cortex of human kidneys ($N = 41$). The isolated membranes were digested by trypsin and the digest was subjected to liquid chromatography–tandem mass spectrometry analysis. The mean expression of surrogate peptides was as follows (given with the standard deviation, in picomoles per

milligram of total membrane protein): OAT1 (5.3 ± 1.9), OAT2 (0.9 ± 0.3), OAT3 (3.5 ± 1.6), OAT4 (0.5 ± 0.2), OCT2 (7.4 ± 2.8), OCTN1 (1.3 ± 0.6), OCTN2 (0.6 ± 0.2), P-glycoprotein (2.1 ± 0.8), MRP2 (1.4 ± 0.6), MRP4 (0.9 ± 0.6), MATE1 (5.1 ± 2.3), and SGLT2 (3.7 ± 1.8). Breast cancer resistance protein (BCRP) and MATE2-K proteins were detectable but were below the lower limit of quantification. Interestingly, the protein expression of OAT1 and OAT3 was significantly correlated ($r > 0.8$). A significant correlation was also observed between expression of multiple other drug transporters, such as OATs/OCT2 or OCTN1/OCTN2, and SGLT2/OCTNs, OCT, OATs, and MRP2. These renal transporter data should be useful in deriving *in vitro* to *in vivo* scaling factors to accurately predict renal clearance and kidney epithelial cell exposure to drugs or their metabolites.

Introduction

Approximately 30% of approved drugs are predominantly (>50% of total body clearance) cleared by the kidneys (Brater, 2002; Feng et al., 2010). Renal drug clearance is the net effect of glomerular filtration plus net tubular secretion (secretion minus reabsorption). Tubular secretion of hydrophilic drug molecules is primarily mediated by both solute carrier and ATP-binding cassette transporters of the proximal tubular cells, located in the renal cortex. For example, the major influx transporters such as organic anion transporters 1 and 3 (OAT1 and OAT3) and organic cation transporter 2 (OCT2) are located in the basolateral membrane, whereas the major efflux transporters such as multidrug and toxin extrusion 1 (MATE1) and P-glycoprotein (P-gp) are located in the apical membrane of the proximal tubules (Feng et al., 2010; Hillgren et al., 2013). These transporters often work in tandem to drive vectorial transport (and therefore secretion) of drugs from the blood into the urine (Meyer zu Schwabedissen et al., 2010). For example, cimetidine and pyrimethamine reduce the renal clearance of metformin by

inhibiting OCT2 and MATE1 or MAT1/2-K, respectively (Wang et al., 2008; Tsuda et al., 2009; Kusuvara et al., 2011; Ito et al., 2012; Feng et al., 2013).

Proximal tubule drug transporters have also been associated with the renal toxicity of drugs (Takeda et al., 1999; Enomoto et al., 2002; Ludwig et al., 2004; Iwata et al., 2012; Moss et al., 2014; Mandíková et al., 2016). For example, cisplatin produces renal toxicity due to its accumulation in the tubular epithelial cells mediated by OCT2. This accumulation occurs because cisplatin is not a good substrate of the kidney epithelial efflux transporters, MATE1 or MATE2-K (Yonezawa and Inui, 2011). However, analogs of cisplatin such as carboplatin and nedaplatin lack significant nephrotoxicity, likely due to their poor affinity for OCT2 (Yokoo et al., 2007).

For the reasons cited above, during the drug development process, it is important to predict whether a drug is likely to be cleared in humans by renal secretion and the extent of the secretion. For instance, if the renal clearance of a drug exceeds its filtration clearance, secretion of the drug by renal transporters should be considered. In addition, it is important to determine whether a drug will accumulate in the kidney epithelial cells. Although *in vitro* tools such as primary two-dimensional cultures or three-dimensional models (organoid models) of renal proximal tubular cells are becoming available or are being developed (Brown et al., 2008; Verhulst et al., 2008; Kelly et al., 2013), they are not currently routinely available or validated. Instead, we and others have proposed a generic

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ABBREVIATIONS: AB, Ardea Biosciences; BCRP, breast cancer resistance protein; DDI, drug–drug interaction; LC, liquid chromatography; LLOQ, lower limit of quantification; MATE, multidrug and toxin extrusion; MRP, multidrug resistance protein; MS/MS, tandem mass spectrometry; NU, Newcastle University; OAT, organic anion transporter; OCT, organic cation transporter; OCTN, organic/carnitine cation transporter; SGLT, sodium-glucose transporter; UPLC, ultra-performance liquid chromatography; UW, University of Washington Medical Center.

in vitro to in vivo extrapolation method for the prediction of transporter-mediated drug disposition, including renal secretory clearance (Prasad and Unadkat, 2014b). This potential approach is based on measurement of in vitro clearance of a drug in cells expressing individual proximal tubule transporters. The in vitro clearance from expressed cell lines is scaled to in vivo clearance using transporter expression levels measured in the human kidney cortex. Since data from the latter are currently limited (Nakamura et al., 2016), this study aimed to quantify proximal tubule-expressed transporters by quantitative targeted proteomics (based on the surrogate peptide approach) and liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS). The selectivity and reproducibility of this approach are discussed herein.

Materials and Methods

Chemicals and Reagents. The ProteoExtract native membrane protein extraction kit was procured from Calbiochem (Temecula, CA). The protein quantification BCA kit and the in-solution trypsin digestion kit were purchased from Pierce Biotechnology (Rockford, IL). Synthetic light and heavy peptides (Supplemental Table 1) for renal transporter quantification were obtained from New England Peptides (Boston, MA) and Thermo Fisher Scientific (Rockford, IL), respectively. High-performance LC-grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ), and formic acid was purchased from Sigma-Aldrich (St. Louis, MO). All reagents were of analytical grade.

Procurement of Kidney Cortices. Noncancerous portions (by pathologic examination) of the human kidney cortex ($n = 20$) from nephrectomies (due to kidney cancer) were collected at the University of Washington Medical Center (UW; Seattle, WA) (Supplemental Table 2). In addition, cortices of kidneys initially targeted for transplant purposes, but eventually not transplanted, were obtained by Ardea Biosciences (AB; $n = 7$ samples; San Diego, CA) and Newcastle University (NU; $n = 14$; Newcastle, UK). To assess whether the transporter expression was sample-site dependent, the kidney cortices of seven subjects, collected by AB, were sampled (approximately 100 mg) from three different locations (top, middle, and bottom of the kidney; Fig. 1). The NU samples were available to us as homogenates in a buffer consisting of 25 mM Tris-HCl, 0.5 mM EDTA, 5 mM histidine, and 0.25 M sucrose, pH 7.4. All of the above samples were immediately flash frozen or stored in ice-cold saline buffer for a maximum of 24 hours prior to storage at -80°C . Tissue collection was approved by the respective human subjects division. Although demographic information was available for the UW and AB samples, such information was not available for the NU samples due to human subject restrictions.

Membrane Protein Extraction and Trypsin Digestion. Total membrane was isolated from the kidney cortex (approximately 100 mg) using a previously described protocol (Prasad et al., 2014). The NU samples were also processed similarly except, instead of the tissue homogenization, a 100- μl aliquot of the cortex homogenate was diluted with 1.9 ml extraction buffer I containing 10 μl protease inhibitor cocktail (ProteoExtract Kit; Calbiochem). The final membrane fraction was diluted to a working concentration of 2 μg membrane protein/ μl as quantified by the BCA assay. Total membrane proteins were reduced, denatured, alkylated, and digested as per our previously reported protocol (Wang et al., 2015). All samples were digested and processed in triplicate. The surrogate peptides generated by trypsin digestion were quantified by LC-MS/MS as described below.

Surrogate Peptide Selection and Quantification by LC-MS/MS. Peptides unique for each transporter and markers of the proximal tubule (proteins predominately located in the proximal tubule) (Supplemental Table 1) were selected based on in silico selection criteria (Kamiie et al., 2008; Prasad and Unadkat, 2014b) and used as calibrators. The corresponding peptides, heavy at [$^{13}\text{C}_6$ $^{15}\text{N}_2$]-lysine and [$^{13}\text{C}_6$ $^{15}\text{N}_4$]-arginine residues, were used as the internal standards. Eight calibrators ranging from approximately 0.1 to 50.0 fmol (on-column) were prepared by spiking the extraction buffer II of the membrane protein extraction kit with the peptide standards and the internal standards (10 μl).

Surrogate peptides were quantified using the Waters Xevo TQ-S tandem mass spectrometer coupled to an Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Hertfordshire, UK). Briefly, a UPLC column (Acquity UPLC HSS T3 1.8 μm , 2.1 \times 100 mm; Waters), with a Security Guard column (C18, 4 mm \times 2.0 mm; Phenomenex, Torrance, CA), was eluted (0.3 ml/min)

with a gradient mobile phase consisting of water and acetonitrile (with 0.1% formic acid; Supplemental Table 1). The injection volume was 5 μl (approximately 10 μg total protein). Optimized LC-MS/MS parameters (Supplemental Table 1) in positive electrospray ionization mode were used to monitor the parent to product ion transitions for the analyte peptides and their respective heavy peptides.

Data Analyses. The data were processed by integrating the peak areas generated from the reconstructed ion chromatograms for the analyte peptides and the respective heavy internal standards using MassLynx software (Waters). For quantification of samples or standards, the peak response from two transitions of each peptide was averaged. As justified in our previous publication (Prasad et al., 2014), the peptide yielding the higher value of transporter expression was reported. Protein expression from different sites (top, middle, and bottom parts) of seven subjects was compared by one-way analysis of variance followed by Bonferroni's multiple comparisons test. A protein-protein expression correlation (r^2) > 0.4 , estimated using the Spearman correlation, was considered significant. Proteins predominantly expressed in the proximal renal tubules (aquaporin 1, dipeptidase 1, arginosuccinate synthase 1, and dicarbonyl-L-xylulose reductase) were also quantified. Since the expression of all of these markers showed high correlation with each other ($r^2 > 0.75$, data not shown), aquaporin 1, which is expressed in high abundance and is a membrane protein, was used as a proximal tubule membrane marker. The Grubbs' test was used to identify outliers (Verma et al., 2014). The transporter protein abundance data are presented as means \pm S.D.

Results

Comparison of Transporter Expression in Cortex Samples Isolated from Different Parts of the Kidney. The expression of all of the transporters was not significantly different between the sampling sites except for the few cortices sampled from the top of subject 4's kidney (Fig. 1). This site-dependent difference (where present) disappeared when transporter protein expression was expressed relative to that of aquaporin 1 (a marker of proximal tubules). This suggests that some of the observed differences (especially for subject 4) were likely due to contamination of the tissue from other parts of the kidney such as the medulla (containing a part of the loop of Henle and the collecting ducts) where the transporters of interest are poorly or not expressed.

Renal Transporter Quantification. Based on surrogate peptides, the expression of OCT2 in the kidney cortex was found to be the highest, followed by that of OAT1, OAT3, and MATE1 (Figs. 2 and 3; Table 1). Although MATE2-K and breast cancer resistance protein (BCRP) were detected in the cortex, they were below the lower limit of quantification (LLOQ) (signal to noise ratio < 5). Compared with the human liver (Deo et al., 2012; Prasad et al., 2014, 2016; Kumar et al., 2015), P-gp and MATE1 expression in the kidney cortex was 2-fold and 10-fold higher than the liver and multidrug resistance protein 2 (MRP2) expression was 2-fold lower than the liver, respectively. Of the transporters quantified, the protein expression showed moderate variability with a percent coefficient of variation of 34%–51% except for MRP4, which was the most variable transporter with a percent coefficient of variation of 70.7%. Using the limited demographic information available for 27 subjects, no correlation was observed between transporter protein expression and age (donors were aged > 40 years), sex ($n = 22$ men and $n = 5$ women), or disease condition such as hypertension ($n = 12$ versus $n = 15$ controls) or diabetes ($n = 7$ versus $n = 20$ controls).

Protein-Protein Correlation of Kidney Transporter Expression. Multiple transporters showed correlation (Supplemental Table 3). Notably, protein expression of OAT1 versus OAT3 showed the highest correlation ($r > 0.8$) (Supplemental Fig. 1; Supplemental Table 3). Other transporters, such as OAT1 versus OAT2, OCT2, or organic/carnitine cation transporter 2 (OCTN2); OAT3 versus OAT2, OCT2, or P-gp; and OCTN1 versus OCTN2 also showed significant correlation

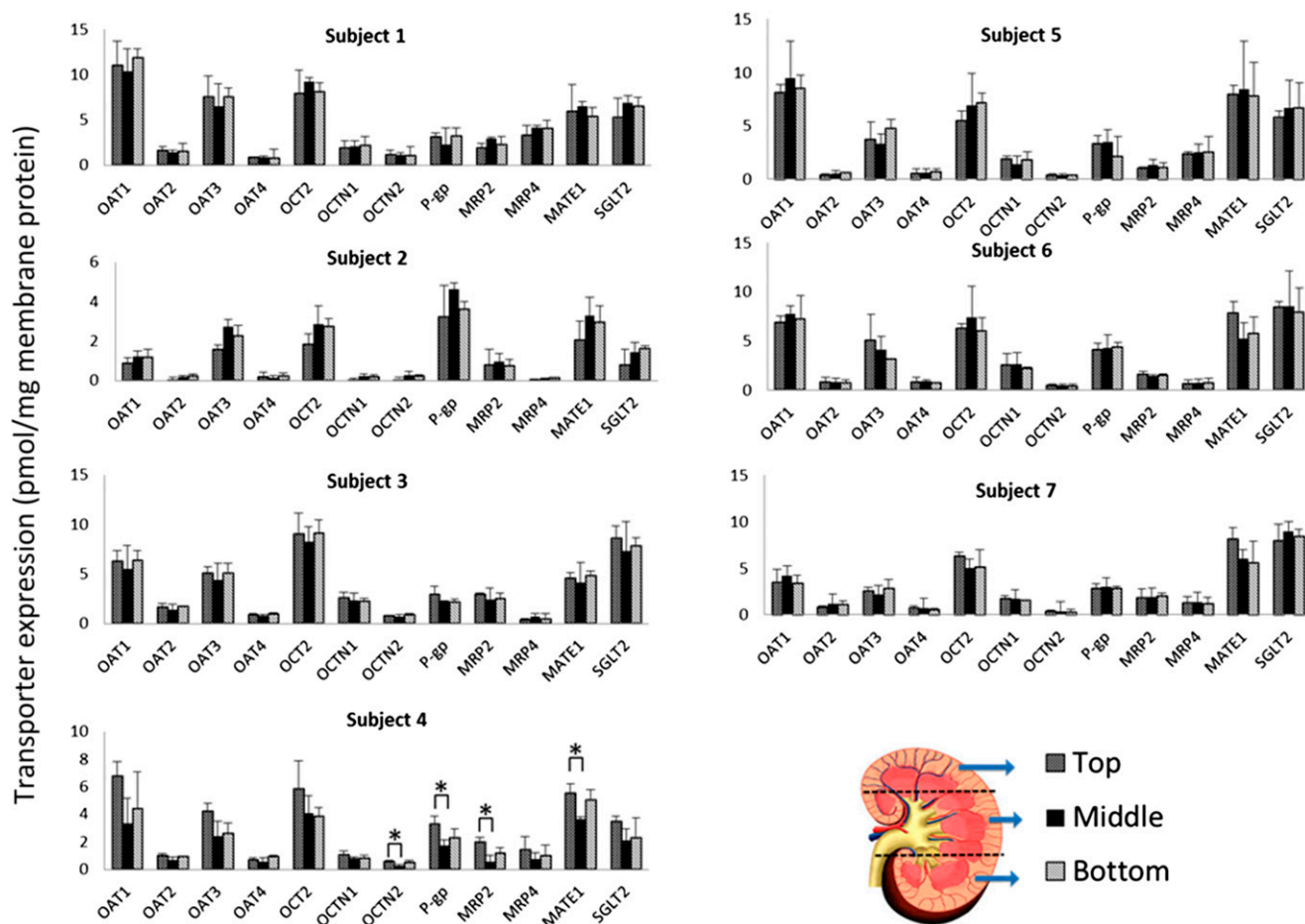


Fig. 1. Transporter protein expression in human cortices obtained from three different parts of the human kidney from seven subjects. Except for the few samples labeled with an asterisk ($*P < 0.05$, analysis of variance followed by Bonferroni's multiple comparison test), transporter protein expression was independent of the sampling site. Data are presented as means \pm S.D. of triplicate determinations.

(Supplemental Table 3). Interestingly, sodium-glucose transporter 2 (SGLT2) expression was correlated with the expression of multiple transporters (Supplemental Table 3).

Discussion

A sensitive and reproducible LC-MS/MS method was developed for the quantification of the polyspecific drug transporters in the human renal cortex (Fig. 1). Because a blank biologic sample matrix was not

available to generate a standard curve, the heavy labeled peptides were used as internal standards to overcome any ion suppression effect produced by the matrix. We have used this approach numerous times to quantify transporter expression in the human liver (Deo et al., 2012; Prasad et al., 2013, 2014, 2016; Prasad and Unadkat, 2014a,b; Kumar et al., 2015). In those studies, we used human liver tissue preparation, spiked with known concentrations of peptides, as quality control samples. These quality control samples (after correcting for the presence of endogenous signal) passed our criteria for accuracy (within 30% of

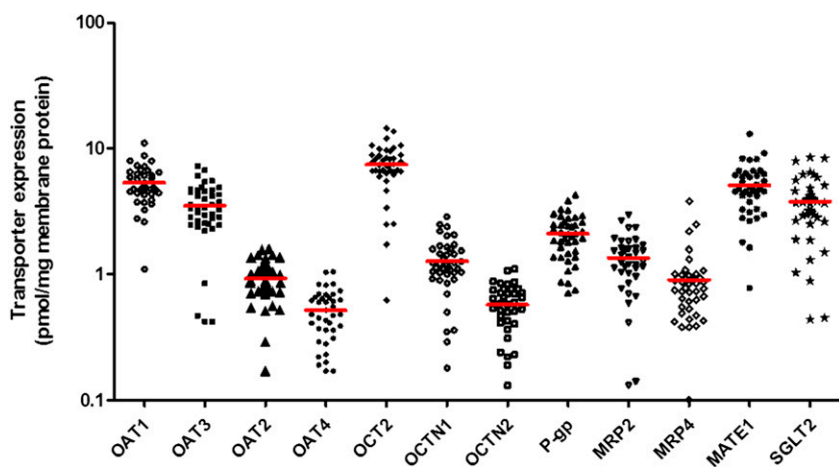


Fig. 2. Interindividual variability in transporter protein expression in the human kidney cortex ($N = 41$). Data points indicate observed data and lines indicates mean expression. Of the transporters quantified, the protein expression of MRP4 was the most variable with a percent coefficient of variation of 70.7%.

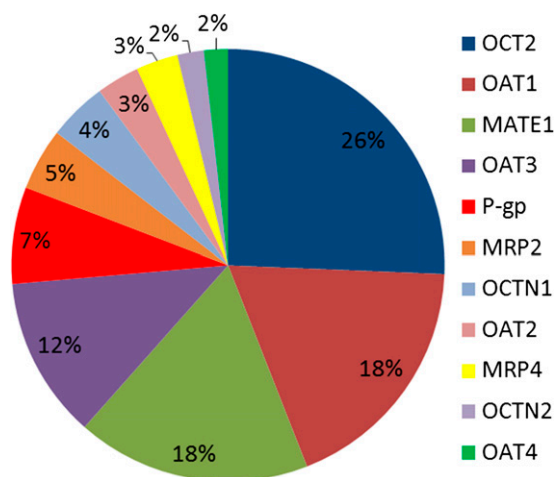


Fig. 3. Relative transporter expression shows that OCT2, OAT1, OAT3, and MATE1 are the predominant transporters expressed in the human kidney cortex.

nominal value), indicating that the tissue matrix did not adversely affect our ability to quantify transporters expressed in these tissues.

We focused our study on quantifying protein expression of renal cortex transporters because these transporters are thought to be predominately located in the proximal tubule of the kidney (Giacomini et al., 2010). Transporter expression was found to be consistent across cortex samples obtained from different parts of the kidney (Fig. 1). These results assure that sampling any part of the kidney cortex will result in similar transporter protein expression and demonstrate the reproducibility of our protein quantification method. Based on peptide quantification, the expression of OCT2 in the kidney cortex was found to be the highest, followed by that of OAT1, OAT3, and MATE1 (Fig. 3). Although we quantified the major drug transporters, our study did not include all transporters such as organic anion-transporting polypeptide 4C1, urate transporter 1, and the thiamine transporters. MATE2-K and BCRP were detected in the cortex, but they were below the LLOQ. Our data can be compared with one other study in which only four transporters (P-gp, MRP2, BCRP, and MRP3) in kidneys from four donors were quantified (Fallon et al., 2016). Our P-gp and MRP2 expression data are similar to those reported in that study (Fallon et al., 2016). Consistent with our data, BCRP abundance was found to be close to our LLOQ (Fallon et al., 2016). MRP3 was not detectable in our study perhaps due to poor sensitivity of the surrogate peptide used (Wang et al., 2015). The low expression of MATE2-K, MRP3, and BCRP in the kidneys does not mean that these transporters are not important in the renal clearance of drugs. If the fraction of a drug transported (f_t) via these transporters versus passive diffusion or transport by other transporters is large, then these transporters will be important in the renal clearance of the drug. As advances in LC-MS/MS instrumentation and protein sample enrichment methods emerge (e.g., capture by anti-peptide antibodies; Razavi et al., 2012), MATE2-K, MRP3, and BCRP may be detected using the surrogate peptides identified in this study.

The mRNA levels of drug transporters in the human kidney have been quantified (Sun et al., 2001; Hilgendorf et al., 2007; Kikuchi et al., 2007; Nozaki et al., 2007). The rank order of mRNA expression is generally consistent with our data on protein expression. The mRNA expression of OAT1 and OAT3 is 5- and 3-fold higher than that of the next highest expressed transporter, OAT4, respectively. The mRNA expression of other transporters in the kidney has the following rank order: MDR1 > MRP2 > OCT2 > OCTN2 > MRP4 (Hilgendorf et al., 2007). As we previously reported, the mRNA expression of transporters does not necessarily correlate with that of the protein (Deo et al., 2012). This lack of correlation could be due to differences in stability, trafficking to the membrane, and/or

TABLE 1

Transporter protein expression in the human kidney cortex

Data are presented as means \pm S.D. given in picomoles per milligram of membrane protein ($N = 41$).

Transporter	Protein Expression	Percent Coefficient of Variation
Uptake		
OAT1	5.33 \pm 1.88	35.3
OAT2	0.93 \pm 0.32	34.6
OAT3	3.50 \pm 1.55	44.3
OAT4	0.52 \pm 0.23	44.5
OCT2	7.42 \pm 2.84	38.3
OCTN1	1.27 \pm 0.59	46.1
OCTN2	0.57 \pm 0.24	41.2
SGLT2	3.67 \pm 1.86	50.6
Efflux		
P-gp	2.09 \pm 0.82	39.3
BCRP	< LLOQ	
MRP2	1.35 \pm 0.63	46.4
MRP4	0.91 \pm 0.64	70.7
MATE1	5.06 \pm 2.27	45.0
MATE2-K	< LLOQ	

regulation (Koussounadis et al., 2015). For this reason, and because protein expression is likely to be more representative of transporter activity, our study focused on measurement of transporter protein expression rather than mRNA expression. However, it is important to note that our methodology does not distinguish between transporter protein expression in the plasma membrane versus that in the intracellular compartments.

Consistent with the reported data on mRNA expression (Nozaki et al., 2007), protein expression of OAT1 and OAT3 was significantly correlated ($r^2 =$ approximately 0.8) (Supplemental Table 3). Taken together, these findings indicate transcriptional coregulation of these transporters. In addition, the expression of other uptake transporters was also correlated, indicating a common mechanism(s) of regulation of these proteins (Supplemental Table 3). To our surprise, SGLT2 protein expression was found to be correlated with the protein expression of most (but not all) transporters (namely, OCTN2, OCTN1, OAT1, OAT2, OCT2, and MRP2). This selective correlation with only some transporters suggests that these correlations were not due to the quality (e.g., contamination from medulla) of the samples. If the latter was the case, SGLT2 protein expression would be correlated with all of the quantified transporters. Transporter protein expression was not correlated with age, sex, or disease state (hypertension or diabetes) in our limited samples. Although sample AB2 with stage 3 kidney disease showed significantly lower expression of the renal transporters, the limited sample demographic information did not allow us to definitively (due to low power) determine the influence of disease conditions on transporter expression. A larger number of kidney samples will need to be analyzed to determine the influence of these covariates on transporter expression. It would be interesting to compare the data presented here with transporter expression in diseased kidneys to predict the effect of kidney failure on the disposition of renally cleared drugs.

The data presented here, together with the affinity of the transporter for a drug, can help quantify the contribution of a transporter in the in vivo renal clearance of a drug. For example, despite the 7.9-fold lower kidney protein expression of OAT2 versus OCT2, based on the higher affinity of OAT2 for creatinine ($K_m = 0.8$ mM for OAT2 versus 18.8 mM for OCT2) (Lepist et al., 2014; Shen et al., 2015), OAT2 is likely to be the major contributor to the in vivo renal secretion of creatinine. This assumes that the V_{max} of the transporter in vivo is proportional to the expression of the transporter in the kidney cortex and the affinity of the drug for the transporter in vitro is equal to that in vivo.

As illustrated by the extended clearance model, inhibition of efflux transporters in the kidney epithelial cells (e.g., MATE1) could result in a

significant increase in drug concentration in these cells (and therefore potential toxicity) without a concurrent change in the concentration of the drug in the systemic circulation (Patilea-Vrana and Unadkat, 2016). That is, these drug–drug interactions (DDIs) cannot be detected by the classic DDI studies in which the plasma concentration of a drug is measured in the presence and absence of an inhibitor. These “silent” drug interactions are a significant challenge in drug development. Although imaging techniques such as positron emission tomography can visualize such DDIs, these studies are not routinely possible. Therefore, alternative methods such as physiologically based pharmacokinetic models, based on transporter expression data, are needed to predict such DDIs. Here, for the first time, we provide such data for the renal cortex transporters in a relatively large sample size. These transporter protein expression and correlation data will be useful in deriving *in vitro* to *in vivo* scaling factors to predict the renal clearance of drugs or their metabolites and their renal tissue concentrations via physiologically based pharmacokinetic modeling and simulations. In addition, these data can be used to predict which transporter may be the rate-determining step in the *in vivo* clearance of the drug and hence predict where a DDI may manifest, such as in the systemic circulation, tissue, or both (Patilea-Vrana and Unadkat, 2016).

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Authorship Contributions

Participated in research design: Prasad, Lee, Kelly, Himmelfarb, Unadkat.
Conducted experiments: Prasad, Johnson, Billington.
Contributed new reagents or analytic tools: Lee, Chung, Brown.
Performed data analysis: Prasad, Johnson, Unadkat.
Wrote or contributed to the writing of the manuscript: Prasad, Johnson, Billington, Lee, Brown, Kelly, Himmelfarb, Unadkat.

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